CXCR7-dependent angiogenic mononuclear cells trafficking regulates tumor progression in multiple myeloma

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KEY POINTS

- Angiogenic mononuclear cells (AMCs) home to tumor sites in multiple myeloma (MM).
- CXCR7 inhibition delayed tumor progression in MM through specific regulation of AMCs trafficking but not through a direct tumor effect.

ABSTRACT

Angiogenesis is a hallmark of progression in multiple myeloma (MM), and many studies have shown that angiogenesis should be considered as a therapeutic target in MM. The CXCR4/SDF-1 axis is essential for cell trafficking and has been shown to regulate tumor progression and metastasis in many tumors including MM. A second chemokine receptor for SDF-1, CXCR7 was discovered recently and is expressed on activated endothelial cells. In this study, we examined the role of CXCR7 in angiogenic mononuclear cells (AMCs) trafficking in MM. Our data demonstrate that AMCs are circulating in patients with MM and in vivo studies show that they specifically home to areas of MM tumor growth. CXCR7 expression is important for regulating trafficking and homing of AMCs into areas of MM tumor growth and neo-angiogenesis. In vitro and in vivo studies confirmed that the CXCR7 inhibitor, POL6926 abrogated trafficking of AMCs to areas of MM tumor progression leading to a significant inhibition of tumor progression in MM. These effects were through regulation of endothelial cells and not through a direct tumor effect. These studies indicate that targeting a bone marrow microenvironmental cell and not direct inhibition of the tumor cells can lead to a delay in MM tumor progression. In conclusion, our studies demonstrate that CXCR7 may play an important role in the regulation of tumor progression in MM through an indirect effect on the recruitment of AMCs to areas of MM tumor growth in the bone marrow niche.
INTRODUCTION

Multiple myeloma (MM) is a plasma cell malignancy that depends on interactions with the bone marrow (BM) microenvironment for growth and survival. In turn, adhesion of MM cells to the BM microenvironment provides a mechanism of resistance to standard chemotherapeutic agents. Angiogenesis is a hallmark of progression in MM, and many studies have shown that angiogenesis should be considered as a therapeutic target in MM. Angiogenic mononuclear cells (AMCs) have been shown in solid tumors to play an essential role in tumor progression by secretion of pro-angiogenic growth factors, and by direct luminal incorporation into sprouting vessels. These cells migrate from the BM to the tumor site through a highly regulated process involving chemotaxis, adhesion and invasion. The BM microenvironment in MM is characterized by an increased micro-vessel density and increased secretion of angiogenic factors.

The CXCR4/ CXCL12 (SDF-1) axis is essential for cell trafficking and has been shown to regulate tumor progression and metastasis in many tumors including MM. It has been previously shown that MM cells are more sensitive to chemotherapy after disrupting their adhesion using a selective CXCR4 antagonist. A second chemokine receptor for SDF-1, CXCR7 was discovered recently. This receptor was previously classified as the orphan G-protein coupled receptor RDC1. It was shown that CXCR7 has two chemokine ligands, SDF-1 and CXCL11, and CXCR7 binds SDF-1 with 10- to 20-fold greater than CXCL11. In landmark studies, CXCR7 surface expression was found on a number of transformed human and mouse cell lines, in addition to activated endothelial cells and embryonic fetal liver cells. Importantly, CXCR7 surface expression was not seen on normal non-transformed tissues despite the presence of CXCR7 mRNA. CXCR7 was found to form functional heterodimers with CXCR4 and enhanced CXCL12-induced signaling. The data also strongly suggested a specialized role for CXCR7 in endothelial biology. There is mounting evidence that CXCR7 itself plays a vital role in cell adhesion, survival and tumor growth, as validated by recent in vitro and in vivo studies. Miao et al showed that CXCR7 overexpression, independent of CXCR4, promoted tumor growth in breast and lung cancer mouse models. These effects were abrogated by CXCR7 knockdown. Taken together, these findings provide a strong rationale for studying the role of CXCR7 in MM. CXCR7 was recently shown to play a key role in AMCs trafficking and angiogenesis.
In this study, we show for the first time that angiogenic mononuclear cells (AMCs) are circulating in patients with MM and specifically home to areas of MM tumor growth. We also demonstrate that CXCR7 expression on AMCs is important for regulating trafficking and homing of AMCs into areas of MM tumor growth and neo-angiogenesis. Inhibition of CXCR7 delays tumor progression through specific regulation of AMCs trafficking and angiogenesis and not through a direct tumor effect.

METHODS

Cells

MM cell lines (MM1.S, U266, RPMI, OPM1 and OPM2) were used in this study. The MM1.S cell line was purchased from ATCC (Manassas, VA), whereas the OPM1 and H929 lines were the kind gift of Prof. Jesús F. San Miguel (Salamanca, Spain). All cell lines were cultured in RPMI-1640 containing 10% fetal bovine serum FBS (Sigma Chemical, St Louis, MO), 2mM L-glutamine, 100 U/mL penicillin, and 100µg/mL streptomycin (GIBCO, Grand Island, NY). The human umbilical vein endothelial cells (HUVECs) (Lonza, Walkersville, MD) were cultured in EGM-2 media (Lonza, Walkersville, MD) and reconstituted according to the manufacturer. MM patient samples were obtained after approval from the Dana-Farber Cancer Institute Institutional Review Board (DFCI IRB). Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Mononuclear cells (MNCs) from the BM and peripheral blood (PB) of MM patients and healthy subjects were obtained by Ficoll (Sigma Aldrich St Louis MO) gradient centrifugation, as previously described. Primary MM cells were obtained using CD138+ micro-bead selection (Milteny Biotec, Auburn, CA).

Reagents

The CXCR7 inhibitor, POL6926 was obtained from Polyphor (Basel, Switzerland). POL6926 is a potent and selective Protein Epitope Mimetic (PEM). It was synthesized with standard fluorenlymethoxycarbonyl (Fmoc) solid-phase strategy on chlorotrityl chloride resin. The peptide, as final form in acetate salt, was purified by preparative reverse phase High Performance Liquid Chromatography (HPLC) and was recovered by lyophilisation. mAbs for flow
Detection of presence of AMCs in BM and peripheral blood (PB) and expression of CXCR7

MNCs from PB of MM patients and normal subjects (n=20 and 5, respectively), MNCs from BM of MM patients and normal subjects (n=5 and 5, respectively); MNCs from BM and PB (n=5 and 5, respectively) of SCID mice (which were injected with 2x10^6 MM1.S cells or vehicle, and tumor growth was allowed for 4 weeks, n=5 for each group), and HUVECs were stained with FITC-anti-CD31 (BD Biosciences, San Jose, CA), PE-Cy7-anti-CD34 (BD Biosciences, San Jose, CA) and anti-CXCR7 (ab72100, Abcam, Cambridge, MA) antibodies for 1hr, followed by a secondary Alexa Fluor 647–conjugated ab (a21244, Invitrogen, Grand Island, NY) for 30min on ice and analyzed by flow cytometry for presence of AMCs and the expression of CXCR7 on a FACS CANTO II flow cytometry system (BD Biosciences, San Jose, CA).

Moreover, MM cell lines were tested for expression of surface and intracellular CXCR7 expression. For surface expression, MM cells were stained with anti-CXCR7 antibodies for 1hr, followed by a secondary Alexa Fluor 647–conjugated ab (a21244, Invitrogen, Grand Island, NY) for 30min on ice and analyzed by flow cytometry for expression of CXCR7. For intracellular expression, MM cells were fixed and permeabilized using Cytofix/Cytoperm Kit (BD Biosciences, San Jose, CA), according to manufacturer’s instructions, cells were then stained with anti-CXCR7 ab for 1hr, followed by a secondary Alexa Fluor 647–conjugated ab (a21244, Invitrogen, Grand Island, NY) for 30min on ice and analyzed by flow cytometry.

Migration in vitro

Migration was performed in trans-well migration assay as previously described. Briefly, HUVECs (5x10^6/ml) were placed in the upper chamber, while the lower chamber had BM supernatant from MM patients or normal subjects; RPMI media or conditioned media from cultures of MM1.S, OPM1 and OPM2 cells lines, migrating cells in the lower chamber were counted by flow cytometry. In some cases HUVECs were pretreated with POL6926 for 3hrs
before the migration assay. Moreover, after the completion of the migration assay, the membrane of the upper chamber was isolated washed, fixed, and the lower side was imaged by a fluorescent microscope.

**Proliferation assay**

MM1.S, OPM2 and HUVECs were treated with increasing concentrations (0-500 nM) of POL6926 for 24hrs, and analyzed by BrdU assay, as previously described. In some cases MM cells were co-cultured with endothelial cells in presence or absence of POL6926 50nM.

**Tube formation assay**

HUVECs were cultured in the presence or absence of MM1.S conditioned media, MM1.S cells, and POL6926 on Matrigel for 8hrs, and tube formation was quantified by light microscopy as previously described.

**Specific homing of HUVECs to MM tumors in vivo**

MM1.S-GFP-Luc (0.5x10^6) cells were injected to the right tibia, while vehicle was injected to the left tibia of 6 SCID mice, as previously described. RFP-HUVECs (0.2x10^6) were injected intravenously to each of 3 mice at days 7 and 14; next day, mice were sacrificed and their tibias were collected, and then BM was analyzed by flow cytometry for the presence of MM cells (GFP+) and HUVECs (RFP+) cells. Animal experiments were approved by Dana-Farber Cancer Institute and Massachusetts General Hospital Institutional Animal Care and Use Committees.

**Effect of POL6926 on homing of HUVECs to MM tumors in vivo**

MM1.S-GFP-Luc (2x10^6) cells were injected intravenously to 6 SCID mice, and allowed to grow for 4 weeks. RFP-HUVECs were treated ex vivo in presence or absence of 50nM POL6926 for 3hrs and cells (0.2x10^6)/mouse were injected to 3 mice in each group. 24hrs after injection, mice were sacrificed and their femurs were collected and their BM was analyzed by flow cytometry for the presence of MM cells (GFP+) and HUVECs (RFP+) cells.
**Effect of POL6926 on number of AMCs in circulation**

BALB/c mice were implanted with Alzet-pumps 1002 (each is loaded with 3.036mg and replaced at after 2 weeks), 2002 (each is loaded with 3.036mg and replaced at after 2 weeks) and 2004 (each is loaded with 6.072mg), each type was implanted into 12 mice. 3 mice were sacrificed and blood was drawn at 0, 7, 14, 21 and 28 days, and circulating AMCs were analyzed by flow cytometry.

**Effect of POL6926 on MM tumor growth**

SCID mice were injected intravenously with MM1.S-GFP-Luc cells. Bioluminescence imaging (BLI) was done twice weekly for 21 days. Mice with similar tumor burden were divided into two groups and implanted with Alzet-pumps-2004 loaded with 0 or 6.072mg of POL6926. Tumor growth was monitored weekly for 3 weeks by BLI.

**β-arrestin assay**

A total of 3 × 103 CHO-CXCR7-βgal1:β-arrestin2-βgal2 (CHO-CXCR7) (DiscoverX 93-0248P2) in 20 µl were seeded into 384-well plates and cultured overnight. The next day, 5 µl of the compounds at varying concentrations of ligands was added to the wells, and the plates were incubated in 5% CO2 at 37°C. After 90 min, 15 µl of β-galactosidase (β-gal) substrate (DiscoveRx) was added to the wells and the plates were incubated at room temperature. After 90 minutes, light emission was analyzed in a Victor II V plate reader (Wallac/PerkinElmer).

**Pharmacokinetic analysis of POL6926**

Samples (50 µL) from all experiments with administration of POL6926 in vivo were spiked with an internal standard, extracted with acetonitrile-2% formic acid, supernatants were evaporated and reconstituted in ACN/DMSO/H2O-50/45/5+2% formic acid; and POL6926 analyzed using HPLC (Acquity UPLC® BEH C18, 100 x 2.1 mm, 1.7 μm, Waters) coupled to mass spectrometry detection (4000 Q Trap mass spectrometer, AB Sciex).
Immunoblotting

HUVEC cells were cultured for 24hrs before the experiment in 6-well plates at 5x104 cells/well, washed with PBS and treated with POL6926 (0 or 50nM) for 6hrs, and MM.1S cells were then applied for 1 hour. Non-activated HUVECs (mono-culture) were used as a negative control. After co-culture MWM cells were separated from HUVEC cells using gentle pipetting, HUVECs were washed with ice-cold PBS, lysed, and protein concentration was normalized. Proteins were blotted using 8-12% acrylamide gels, transferred to a nitrocellulose membrane; membranes were blocked with 5%-nonfat dry milk in TBS/T buffer and incubated with primary antibodies for p-FAK, p-p130, p-Akt, pPI3K-85, pERK, and or α-tubulin overnight at 4°C. The membranes were then washed, incubated with appropriate HRP-conjugated secondary antibody, washed, and developed using luminol base assay. Luminescence was measured using x-ray films.

RESULTS

MM-derived AMCs present with higher expression of CXCR7 in MM compared to healthy individuals

We first examined the number of circulating AMCs and CXCR7 expression on AMCs obtained from both MM patients and MM-harboring mice. Figure 1A shows representative analysis of AMCs by flow cytometry in which AMCs were defined as CD31+/CD34+, as previously described. We found that the presence of AMCs was significantly higher in the BM and PB of MM patients compared to healthy subjects (Figure 1B): the number of AMCs in MM was 3 and 6-fold higher than controls in BM and PB, respectively. Similar results were obtained in the BM and PB of mice engrafted with MM cells compared to control mice that were not engrafted with human MM cells: the number of AMCs in MM mice was 8 and 7-fold higher than control mice in BM and PB, respectively (Figure 1C). We further tested the expression of CXCR7 on the surface of AMCs and compared it to the expression levels on MNCs in BM and PB of MM patients and healthy subjects. We found that AMCs had a high expression of CXCR7 compared to the rest of the MNCs in both BM and PB. We also found that the surface expression of CXCR7 in AMCs in the BM was higher than the expression in the PB, while there was no difference in CXCR7 expression between AMCs isolated from MM patients or from healthy
subjects (Figure 1D) which suggest disease status does not change CXCR7 surface expression on AMCs.

AMCs home to MM-enriched bone marrow niches

Because of their angiogenic cells-like features, we used HUVECs as representative cells for angiogenic cells in our models. We found that HUVECs have high surface expression of both CD31+ and CD34+, as well as high expression of CXCR7 (Figure 2A). These cells were found to migrate to BM-supernatant from MM patients with 3-fold higher rate of migration compared to BM-supernatant from normal subjects (Figure 2B). These results were confirmed in vitro, in which HUVECs migrated to MM cell-line (MM1.S, OPM1 and OPM2) conditioned media about 2-fold higher than non-conditioned media (Figure 2C). To test the specificity of the homing of AMCs to MM tumors in vivo, we injected MM cells in the right tibia and vehicle control in the left tibia and MM cells were allowed to grow for 2 weeks. Then, HUVECs were injected into the mice at 1 and 2 weeks after injection of MM cells, and the homing of HUVECs to the right and left tibias as well as the MM cell percentage in the BM were analyzed 24hrs after injection of the HUVECs (Figure 2D represents a schematic description of the procedure). The growth of MM cells was higher in the right tibia at 1 and 2 weeks compared to the left tibia, indicating specific tumor growth, while some MM growth was observed in the left tibia at 2 weeks but to a lower extent compared to the right tibia (Figure 2E). HUVECs homed to the right tibia (that had more MM cells) about 2.5 fold higher compared to homing to the left tibia (Figure 2F), suggesting that AMCs home specifically to areas of MM tumor growth.

CXCR7 inhibition decreases the number of AMCs in circulation

To study the role of CXCR7 in regulating endothelial cell trafficking, we examined the effect of the specific CXCR7 inhibitor POL6926, a protein epitope mimetic, on the circulating number of AMCs in healthy SCID-bg mice, and found that a continuous inhibition of CXCR7 delivery of POL6926 with osmotic pumps over 4 weeks (4mg/kg/day s.c.) showed a significant decrease in the number of circulating AMCs in mice over 4 weeks. The three different types of pumps (1002, 2002 and 2004) showed similar patterns of reduction of the AMCs numbers (Figure 3A).
Pharmacokinetic analysis showed that, as expected, the concentration of POL6926 correspond to a plateau around 300nM for the three different types of pumps over the course of the treatment (4 weeks), (Figure 3B). These studies indicate that continuous CXCR7 inhibition inhibits cell trafficking of AMCs.

**CXCR7 inhibition decreases AMC -enhanced MM tumor growth**

To study the role of AMCs in enhancing MM tumor growth and the role of CXCR7 in this process, mice were injected intravenously with MM1.S-GFP-Luc cells ($3 \times 10^6$) and were randomized into two groups with the same mean BLI after 21 days. Then, mice treated with POL6926 by subcutaneous implantation of osmotic pumps (2004) which delivered 4mg/kg/day in each mouse for 21 days; vehicle loaded pumps were implanted in a different group as a control. Mice treated with the CXCR7 inhibitor demonstrated a significant delay in tumor progression compared to the vehicle control group as shown in quantification of BLI (Figure 3C) and in representative images of the BLI (Figure 3D). Analysis of the blood concentration of POL6926 over the treatment period showed that blood levels were well above the concentration needed for inhibition of CXCR7 (Figure 3E). POL6926 potentially targets CXCR7 by recruiting beta-arrestin with an EC50 of 4.6 nM without significantly modulating the activity of CXCR4 up to 10 µM (Figure 3F).

**CXCR7 blockade exerts indirect inhibition of MM**

To investigate detection of CXCR7 expression in MM, flow cytometry was performed in a panel of MM cell lines showing lack of CXCR7 surface expression, and low CXCR7 intracellular expression (Figure 4A). Moreover, the effect of CXCR7 inhibition on MM was tested with POL6929, which showed no effect on proliferation of MM cells in vitro (Figure 4B). Similarly, POL6929 did not modulate the proliferation rate of HUVEC (Figure 4B) In contrast; the compound inhibited the proliferation of MM cells cultured in presence of HUVEC suggesting its ability to overcome HUVEC-dependent growth advantage on MM cells (Figure 4C). Given that POL6926 does not inhibit the proliferation of tumor cells or HUVEC cells, we hypothesized that
this in vivo inhibition of proliferation effect observed in Figure 3D is due to a specific effect of this agent on cell trafficking of AMCs into areas of MM proliferation.

**CXCR7 inhibition modulates homing of AMCs to MM-enriched bone marrow niches**

Prior studies have shown that trafficking of AMCs enhance tumor growth, therefore, we sought to examine the role of AMCs in their specific homing into areas of MM tumor growth as a mechanism for the inhibition of tumor growth observed in our in vivo mouse model. Based on this hypothesis, we first examined the effect of POL6926 on the migration of HUVECs to conditioned media from MM1.S cell line, and found that it inhibited the migration of HUVECs in a dose-dependent manner, with almost full abrogation of the migration at 50nM (Figure 5A). These results were confirmed by fluorescent imaging of the lower side of the migration filter, which showed similar results of inhibition of the migration of HUVEC at 50nM of POL6926 (Figure 5B). Similarly, POL6926 inhibited the migration of HUVECs to BM supernatant from 3 different MM patients to less than 40% of the non-treated cells (Figure 5C).

To further confirm the role of CXCR7 in modulating the homing of AMCs to MM-colonized bone marrow niches, POL6926 was tested in vivo to examine its activity on delaying/preventing specific homing of endothelial cells to areas of MM tumor growth. First, MM cells were allowed to grow for 4 weeks in SCID-bg mice by intravenous injection of GFP-Luc+MM1S (2x10^6) and monitoring of tumor growth using BLI for 4 weeks. HUVECs were then treated in vitro in presence or absence of POL6926 (50 nM) for 3hrs, washed, and injected intravenously into the mice, and the homing of HUVECs to the BM was analyzed after 24hrs using flow cytometry. The schematic description of the procedure is shown in Figure 5D. The MM tumor burden in the bone marrow of the two groups was found to be similar in the two groups of MM bearing animals (Figure 5E). Homing of POL6926-treated HUVECs to the MM tumor regions was significantly lower compared to the non-treated group (Figure 5F). These results indicate that CXCR7 plays an important role in the cell trafficking and homing of AMCs to MM tumors. Therefore, based on this data, we postulate that the mechanism by which the CXCR7 inhibitor decreased tumor growth in the MM tumor model is due to decreased cell trafficking and an
inhibition of specific homing of AMCs to areas of MM tumor growth, leading to a delay in tumor progression.

**CXCR7 regulates endothelial vessel formation and angiogenesis.**

To further confirm that neo-angiogenesis within the bone marrow is the major mechanism by which CXCR7 inhibition regulates tumor growth, we examined the effect of CXCR7 inhibition on vessel formation of HUVECs. We tested the effect of MM cell-derived conditioned media (CM) on tube formation of HUVECs, using Matrigel; and found that MM CM induced significant increase in tube formation, while the effect was abolished by the treatment of HUVECs with the CXCR7 inhibitor, POL6929 (Figure 6A). Relative quantification of tube length formation is provided in Figure 6B. We next evaluated MM cells-dependent ability to increase tube formation in presence or absence of POL6929; and found that the compound was able to inhibit MM-cell induced tube formation of HUVECs (Figure 6C). Activation of HUVECs with MM cells increased the proliferative (pPI3K-P85 and pAKT) and cytoskeletal (pP130 and pFAK) pathways in HUVECs, while inhibition of CXCR7 with POL6926 decreased this activation of these pathways. In addition, CXCR7 inhibition increased the phosphorylation of pERK (Figure 6D).

In summary, these studies demonstrate that AMCs play a critical role in tumor progression in MM and that CXCR7 expression on AMCs is important for regulating trafficking and homing of AMCs into areas of MM tumor growth and neo-angiogenesis. This indicates that inhibition of CXCR7 delays tumor progression through the specific regulation of AMC trafficking and angiogenesis and not through a direct tumor effect.

**DISCUSSION**

Recent studies have shown that the scavenging receptor CXCR7 enhances metastasis complementary to CXCR4. Treatment with a small-molecule inhibitor of CXCR7 chemokine limited the growth of CXCR4+ breast cancer cells in tumors that also contained malignant CXCR7+ cells. In addition, upregulation of CXCR7 in gastric MALT lymphomas has been
associated with progression to diffuse large B cell lymphoma, indicating that this chemokine receptor is also critical in tumor progression in B-cell malignancies.\textsuperscript{29} However, its role in tumor progression in MM has not been previously examined. Our study demonstrates that CXCR7 has a role in tumor progression and cell dissemination in MM but not through a direct activity of the CXCR7 receptor in MM cells but rather through indirect recruitment and activity of AMCs and vasculogenesis in MM. This is consistent with plasma cell biology of CXCR7 null mice where there is little effect on B cell composition.\textsuperscript{15}

Prior studies have shown that angiogenesis is critical during tumor progression in MM with an increase in mean vessel density (MVD) in the bone marrow of patients with MM during tumor progression.\textsuperscript{30-32} Endothelial progenitor cells (EPCs) have been examined in 31 MM patients and were shown to increase in a number of patients with active disease compared to healthy control. Together, these studies indicate that angiogenesis and endothelial cells play an important role in tumor progression in MM. However, the exact mechanisms of regulation of these cells and whether targeting them with a therapeutic agent can have an effect in delaying progression have not been previously examined.

The precise functional role of CXCR7 has remained elusive. While Burns et al\textsuperscript{12} confirmed that the SDF-1 ligand (CXCL12) binds to CXCR7 with high affinity, it was not found to induce calcium mobilization or cell migration as expected for a G-protein coupled chemokine receptor. The role of CXCR7 as a partner (or interceptor) for CXCR4 is also being elucidated. Balabanian et al\textsuperscript{11} found that blocking both CXCR4 and CXCR7 resulted in an additive inhibitory effect on T cell migration mediated by SDF-1. This suggested that the two chemokine receptors may act in concert. However, another possibility proposed by Sierro et al\textsuperscript{15} is that CXCR7 may not function alone but may modulate CXCR4 function via heterodimerization. In support of this, preformed dimers of CXCR4/7 were seen on the surface of HEK293 transfected cells by false-color merged image (FRET). In turn, Ca\textsuperscript{+} flux was enhanced with CXCR4/7 co-expressed cells. Co-expression also affected pERK signaling pattern, resulting in delayed but sustained pERK activation compared to singly expressing CXCR4 cells.\textsuperscript{15} Although it was shown that CXCR7 forms heterodimers with CXCR4 in vitro, heterodimerization in vivo remains to be demonstrated.\textsuperscript{33}
Recently, Mazzinghi et al\textsuperscript{34} showed that CXCR4 and CXCR7 have differential roles in the homing of human renal progenitor cells. SDF-1 induced migration of renal progenitor cells was dependent only on CXCR4, while trans-endothelial migration required both CXCR4 and CXCR7.\textsuperscript{34} Interestingly, POL6926 and other recently published CXCR7 targeting agents e.g. “Compound 1”\textsuperscript{35} and CXCR7 modulators from Chemocentryx Inc., act as CXCR7 agonist for the beta-arrestin recruitment\textsuperscript{36} and were described as functional antagonists\textsuperscript{35,37} by scavenging CXCL12 and negatively regulating CXCL12 functions, suggesting that CXCR7 agonists may have a therapeutic benefit in pathological conditions where CXCL12 is involved.

In this study, we found that the level of AMCs was elevated in the PB and BM of MM patients compared to normal subjects, a finding which was confirmed in a MM mouse model in which CXCR7 was highly expressed on AMCs. Injection of a specific CXCR7 antagonist for a prolonged period of time (4 weeks) decreased the numbers of AMCs in the peripheral blood. Moreover, in vitro and in vivo studies confirmed that the CXCR7 inhibitor POL6926 abrogated trafficking of AMCs to areas of MM tumor progression leading to a significant inhibition of tumor progression in MM. These results demonstrate that CXCR7 has a role in the cell-trafficking and recruitment of AMCs in MM. Our results also indicate that targeting a bone marrow microenvironmental cell and not direct inhibition of the tumor clone can lead to a delay in MM tumor progression. Although prior studies have shown that anti-angiogenic drugs had limited activity on tumor growth in MM,\textsuperscript{38,39} our studies suggest that targeting AMCs before their recruitment into areas of tumor progression may provide a window of opportunity for targeted anti-angiogenic therapy or AMC-targeted therapy that delays/prevents tumor progression. Further studies into the mechanisms of AMC-mediated tumor progression in MM and their specific role in inducing tumor progression are warranted.

The role of CXCR7 antagonists remains to be elucidated in patients with hematological malignancies specifically in MM. CXCR4 inhibitors have been used as stem cell mobilizers or as chemosensitizers in blood cancers including multiple myeloma.\textsuperscript{40-42} Based on our preclinical studies, we believe that CXCR7 inhibitors are critical for regulating the interaction of endothelial cells with tumor cells and preventing ongoing angiogenic signals that lead to tumor progression.
and dissemination. Therefore, early interventions with CXCR7 inhibitors in patients with precursor conditions such as smoldering myeloma or in minimal residual disease states post-transplant are likely to be the most critical time points for therapeutic interventions with agents that disrupt the dependency of tumor cells on microenvironmental cells. Most importantly, we do not believe that agents such as CXCR7 inhibitors should be used as single agents in cases where the tumor cells are not dependent on the bone marrow microenvironment as in extramedullary involvement or plasma cell leukemia.

In conclusion, our studies demonstrate that CXCR7 is a crucial regulator of tumor progression in MM through an indirect effect on the recruitment of AMCs to areas of MM tumor growth in the bone marrow niche.
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AUTHORSHIP

AKA and IS: Performed research, designed research, analyzed data and wrote the manuscript.

FA, MM, YM, AR, NB: Performed research, analyzed data.

KPA, BRO, JZI, EC: provided the CXCR7 inhibitor, PK studies, analyzed data and revised the manuscript.

IMG: designed research, revised the manuscript the paper and supervised the study.

DISCLOSURE OF CONFLICTS OF INTEREST

IMG is on the advisory board for Onyx, Millennium, BMS and Celgene and receives research lab support from Genzyme/Takeda, BMS and Noxxon.

KPA, BRO, JZI, EC is an employee of Polyphor Ltd.

All other authors declare no competing financial interests.
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FIGURE LEGENDS

FIGURE 1: AMCs are more prominent in MM BM and PB and express high CXCR7
(A) Representative images for gating of AMCs (CD31+/CD34+) and MNCs (CD31-/CD34-) populations (left), and diagram of expression of CXCR7 on each of these populations (right). (B) Presence of AMCs expressed as % of total MNCs in the BM and PB of MM patients and normal subjects. (C) Presence of AMCs expressed as % of total MNCs in the BM and PB of mice with and without MM1.S xenografts. (D) The expression of CXCR7 on AMCs from BM and PB isolated from MM patients and healthy subjects, normalized to mean fluorescence intensity (MFI) of MNCs in each sample.

FIGURE 2: AMCs home specifically to MM-enriched bone marrow niches
(A) Expression of CD31, CD34 and CXCR7 on HUVECs, as a model for AMCs. (B) Migration of HUVECs to BM supernatant from normal subjects and MM patients normalized to the average of migration of normal subjects. (C) Migration of HUVEC cells to conditioned culture media from MM cell lines which were incubated in the media for 24hrs before the migration test, normalized to migration to non-conditioned culture media. (D) A schematic description of the procedure. (E) MM tumor progression in the right and left tibias after intra-tibial injection of MM1.S cells into the right tibia. (F) Differential homing of HUVECs to tibias with higher involvement of MM after intravenous injection.

FIGURE 3: Inhibition of CXCR7 prevents the AMC-mediated MM tumor growth
(A) Number of circulating AMCs at 0, 7, 14, 21 and 28 days after subcutaneous implantation of Osmotic Alzet Pumps 1002 (replaced at day 14), 2002 (replaced at day 14) and 2004 loaded with POL9626 in healthy SCID-bg mice. (B) Pharmacokinetic analysis of the levels of POL9626 in plasma over 4 weeks after implantation of the different pumps. The effect of POL9626 (delivered by 2004 osmotic pump) on MM tumor growth by bioluminescent imaging (BLI) as detected at 28, 35 and 42 days after injection of MM cells; as shown in quantification of BLI (C) and images (D). (E) Pharmacokinetic analysis of the levels of POL9626 in plasma of the
Compound 1 and POL6926 triggered association of beta-arrestin with CXCR7 in a concentration-dependent manner.

**FIGURE 4: Inhibition of CXCR7 exerts indirect inhibition of MM**

(A) The expression of CXCR7 on the surface (upper panel) and intracellular (lower panel) of MM cell lines (MM1.S, OPM1, OPM2, H929, RPMI, U266, MM1R and MOLP8). (B) The effect of a range of concentrations (0-500nM) of POL9626 on proliferation of MM1.S, OPM2 and HUVECs when cultured each alone. (C) The effect of POL9626 (50nM) on MM tumor proliferation induced by co-cultured with HUVECs.

**FIGURE 5: Inhibition of CXCR7 disrupts the homing of AMCs to MM-enriched BM niches**

(A) The effect of increasing concentrations (0-500nM) of the CXCR7 inhibitor POL6926 on migration of HUVECs to conditioned media from MM1.S cells (24hrs incubation) normalized to the migration to non-conditioned media as counted by flow cytometry. (B) Representative images of the effect of POL6926 (50nM) on migration of AMCs to conditioned MM1.S media as detected by fluorescent microscopy. (C) The effect of POL6926 (50nM) on migration of HUVECs to BM supernatant from three MM patients, counted by flow cytometry and normalized to migration of non-treated HUVECs in each. (D) A schematic description of the procedure. (E) The MM tumor burden of the two groups of MM-bearing mice. (F) The effect of CXCR7 on the homing of HUVECs, after intravenous injection, to the BM of mice with established xenografts of MM1.S cells.

**FIGURE 6: Inhibition of CXCR7 reverses tube formation induced by MM**

(A) Representative images of the effect of POL9626 (50 nM) on the HUVEC tube formation induced by conditioned media from MM1.S cells, non-conditioned media was used a control. (B) Quantification of the length of tubes formed due to MM conditioned media with or without the presence of POL9626 (50 nM), normalized to the length of tubes formed in non-conditioned media. (C) Representative images of the effect of POL9626 (50 nM) on the tube formation of
HUVEC cells induced by co-culture with MM1.S cells and the direct cell-adhesion of MM cells to the formed tubes. (D) Following activation of HUVEC cells with MM cells, the effect of CXCR7 inhibition on downstream signaling in HUVECs cells was shown by immunoblotting.
Figure 1

A

B

C

D

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Figure 3

A

Circulating AMCs (% of MNCs)

Time (Days)

B

Concentration Average (ng/ml)

Time (Days)

C

Tumor Growth by BLI (Photon/sec/cm²)

Time (Days)

D

Vehicle

POL6926

Day 21

Day 28

Day 35

Day 42

E

Concentration Average (ng/mL)

Time (days)

F

β-arrestin signal

Log [sample] (M)
Figure 4

A

Surface Expression

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Intracellular Expression

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B

Proportion of proliferation (% of control)

- HUVEC
- MM1S
- OPM2

0 10 50 100 250 500

POL6926 (nM)

C

Proportion of MM cells (% of MM cells alone)

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Co-Culture of MM With HUVEC
Figure 6

A

10% FBS Media

10X

MM Media

Vehicle

POL6926 50nM

10X

10X

B

Vessel formation (% of 10% FBS Media)

Vehicle

POL6926 50nM

MM media

10% FBS Media

Vehicle

POL6926 50nM

C

10% FBS Media

20X

MM Cells

Vehicle

POL6926 50nM

20X

20X

D

50nM POL6926

- - + +

MM.1S

- + +

pAKT

pPI3K-pP85

pP130

pFAK

pERK

Tubulin

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CXCR7-dependent angiogenic mononuclear cells trafficking regulates tumor progression in multiple myeloma

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